Listeria monocytogenes in milk products

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Abstract

Milk and milk products are frequently identified as vectors for transmission of *Listeria monocytogenes*. Milk can be contaminated at farm level either by indirect external contamination from the farm environment, or less frequently by direct contamination of the milk from infection in the animal. Pasteurisation of milk will kill *L. monocytogenes*, but post-pasteurisation contamination, consumption of unpasteurised milk and manufacture of unpasteurised milk products can lead to milk being the cause of outbreaks of listeriosis. Therefore, there is a concern that *L. monocytogenes* in milk could lead to a public health risk. To protect against this risk, there is a need for awareness surrounding the issues, hygienic practices to reduce the risk and adequate sampling and analysis to verify that the risk is controlled. This review will highlight the issues surrounding *L. monocytogenes* in milk and milk products, including possible control measures. It will therefore create awareness about *L. monocytogenes*, contributing to protection of public health.

Key-words: *Listeria monocytogenes*, milk, cheese, occurrence, persistence, outbreak

1. Introduction

1.1 *Listeria monocytogenes* in milk and milk environment

As a food, milk is nutritious and contains protein, fats and micro-nutrients that can contribute to the daily diet. Aseptically drawn raw milk is practically sterile. But during the process of milking it can become contaminated by bacteria which come from clinical and sub-clinical infections of the udder or from the farm environment, entering the milk via the teat skin, the milking equipment and the milker. Milk processing can introduce another opportunity for bacteria to contaminate the milk product, i.e. cross-contamination from the processing environment. Most of these bacterial contaminants are harmless but a minority can be disease causing bacteria, like *Listeria monocytogenes*.

L. monocytogenes is the causative agent of listeriosis in humans that can affect susceptible populations such as newborn children, the elderly and immunocompromised persons (Lorber 1997; McLauchlin et al. 2004). Although not very frequent, listeriosis has a mortality rate of 20-30% (Sleator et al. 2009), making it a pathogen of concern for the dairy industry (Table 1).

1.2 *L. monocytogenes* virulence

Currently, the regulations assume that all *L. monocytogenes* are equally pathogenic. This is reasonable as at present there is no test to distinguish between *L. monocytogenes* of different pathogenicity. *L. monocytogenes* expresses about 50 molecules, including the virulence genes listeriolysin O and two major internalins, InlA and InlB, to promote its cell infection cycle (Camejo et al. 2011). Studies have shown that a significant proportion of *L. monocytogenes* isolates from foods carry unique virulence-attenuating mutations in the gene *inl*A, or other virulence factors (Nightingale et al. 2008; Témoin et al. 2008; Van Stelten and Nightingale 2008; Camejo et al. 2011; Chen et al. 2011). In the case of *inl*A, these mutations can be the cause of a premature stop codon which leads to the production of a truncated and secreted InlA but can also completely abolish InlA production, reducing the ability of *L. monocytogenes* to invade human cells (Nightingale et al. 2008). In the future, it may be possible to determine a risk level associated with strains that are lacking certain genes or in which the presence of virulence-attenuating mutations diminishes the virulence potential of the strains. At present such risk assessment is not practiced and it is understood that all strains of *L. monocytogenes* are capable of causing disease.

Table 1. Outbreaks of foodborne listeriosis.

Year	Place	No. of cases (No. of deaths)	Type of milk product	Serotype	Reference
United States of America					
1979	Maryland	20 (3)	Raw milk cheese	4b	Ho et al. (1986)
1983	Maryland	49 (14)	Pasteurised milk	4b	Fleming et al. (1985)
1985	California	142 (48)	Mexican-type cheese	4b	Linnan et al. (1988)
1986-1987	Pennsylvania	36 (44)	Ice cream, brie	4b, 1/2b, 1/2a	Schwartz et al. (1989)
1987	California	11	Butter		FDA (2003)
1994		45	Chocolate milk	1/2b	Dalton et al. (1997)
2000	North Carolina	12 (5)	Queso Fresco cheese	4b	CDC (2001); MacDonald et al. (2005)
2003	Texas	13 (2)	Mexican type fresh cheese		Swaminathan and Gerner-Smidt (2007)
2003-2007	Texas + 7 states	74 (10)	Queso Fresco		Smith (2008)
2007	Massachusetts	5 (3)	Pasteurised milk		CDC (2008)
2008	Multi-state	8	Mexican-style cheese		Jackson et al. (2011)
2009	Washington	2	Cheese		Anonymous (2009a)
Europe					
1981	England	11 (5)	Dairy products	1/2a	FDA (2003)
1983-1987	Switzerland	122 (33)	Soft cheese	4b	Bula et al. (1995)
1986	Austria	28 (5)	Unpasteurised milk		Allerberger and Guggenbichler 1989)
1989-1990	Denmark	26 (6)	Blue-mold cheese/hard cheese	4b	Jensen et al. (1994)
1993	Scandinavia	1	Goat's milk cheese		Eilertz et al. (1993)
1995	France	37 (11)	Soft cheese	4b	Goulet et al. (1995)
1997	France	14	Soft cheese	4b	Jacquet et al. (1998)
1998-1999	Finland	25 (6)	Butter	3a	Lyytikainen et al. (2000)
1999	England	2	Cheese	4b	Kimball (2006)
2001	Sweden	120	Soft cheese	1/2a	Carrique-Mas et al. (2003)
2001	Belgium	2	Frozen ice cream		Yde and Genicot (2004)
2005	Switzerland	10 (3)	Soft cheese	1/2a	Bille et al. (2006)
2006	Czech Republic	75	Cheese	1/2a	Vit et al. (2007)
2006-2007	Germany	189	Acid curd cheese		Koch et al. (2010)
2009-2010	Austria, Germany, Czech Republic	Two outbreaks: 14 (5) and 20 (3)	Quargel cheese	1/2a (2 clones)	Fretz et al. (2010)
Canada					
2001	Manitoba	7	Cream		Pagotto et al. (2006)
2002	British Columbia	47	Cheese	4b	Pagotto et al. (2006)
2002	British Columbia	86	Pasteurised cheese	4b	Pagotto et al. (2006)
2002	Quebec	17	Soft and semi-hard cheese	4b	Gaulin et al. (2003)
2008	Quebec	38 (2)	Cheese	1/2a	Gilmour et al. (2010)
Asia					
2001	Japan	38	Washed-type cheese	1/2b	Makino et al. (2005)
South America					
2008	Chile	119 (5)	Brie		Anonymous (2009b)

1.3 Regulations for *L. monocytogenes* in foods

In order to protect public health, authorities have set regulations on the maximum number of *L. monocytogenes* that can be present in food. In the United States of America there is 'zero tolerance' of *L. monocytogenes*, where any occurrence in food is considered an offence. In the European Union, there is a more lenient approach which is based on the fact that *L. monocytogenes* is ubiquitous in the environment and that <100 cfu/g is insufficient to cause illness. There is a 'zero tolerance' for special dietary foods and foods intended for infants. The regulations also differentiate between foods that can and cannot support growth of *L. monocytogenes*. For foods that cannot support growth, up to 100 cfu/g are allowed in the food during its shelf-life. For foods that can support growth absence is required. It is the responsibility of the food business operator to demonstrate that a food is unable to support growth, otherwise the assumption is that growth will occur and the regulation of absence is applied. In New-Zealand and Australia, the absence of *L. monocytogenes* in 25 grams or millitres for five samples is required throughout the shelf-life for raw milk and raw milk products, and cheeses supporting *L. monocytogenes* growth (Anonymous 2012).

1.4 Scope of the chapter

The scope of this chapter is to assess current knowledge on *L. monocytogenes* occurrence in milk and milk products. It also focuses on the occurrence and persistence of *L. monocytogenes* in milk processing environment, and the strategies to prevent and control it.

2. *L. monocytogenes* contamination of milk

Generally, where *L. monocytogenes* is detected in raw milk the source is not identified, although it most likely comes from the environment post-milking. Studies of *L. monocytogenes* contamination of raw milk found that animal faeces, poor quality feed and general lack of hygiene on the dairy farm are factors associated with contamination (Sanaa et al. 1993; Nightingale et al. 2005; Schoder et al. 2011). Direct excretion of *L. monocytogenes* into the milk, i.e., from clinical or sub-clinical mastitis, is rare although cases where sub-clinical mastitis in cows can go undetected have been reported (Gitter et al. 1980; Van Daelen and Jaartsveld 1988; Fedio et al. 1990; Bourry et al. 1995; Schoder et al. 2003; Winter et al. 2004) (Fig.1).

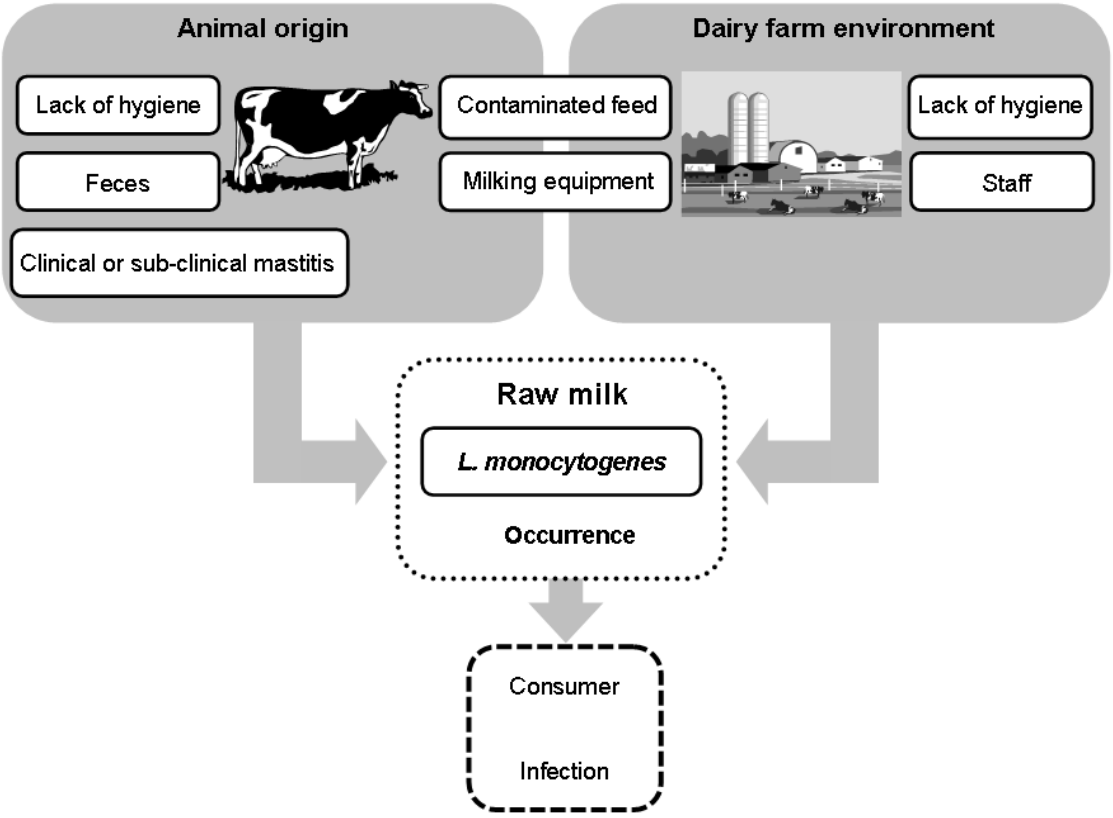

Fig. 1. Flow-chart of contamination of raw milk with *L. monocytogenes*.

2.1. Occurrence of *L. monocytogenes* in milk

The reported incidence of *L. monocytogenes* in milk varies. In Ireland the incidence was 3% (Rea et al. 1992), and 6% (Fox et al. 2011a), in Northern Ireland 8.8% (Harvey and Gilmour 1993), in the USA 19.7% (Latorre et al. 2009), 1% (Waak et al. 2002), 4.1% (Rohrbach et al. 1992) and 0% (D'Amico and Donnelly 2010). Other reported incidences include 1.7% in Iran (Mahmoodi 2010), 13% in Mexico (Vázquez-Salinas et al. 2001) and 2.2% in Turkey (Aygun and Pehlivanlar 2006). Seasonal variation in reported incidence has been observed, although there is no consistency in the variation observed. Waak et al. (2002) reported higher incidence in winter and Vázquez-Salinas et al. (2001) reported a spring/summer peak. Because of the variety of analytical methods used, and the improvements in detection methodologies over the years, it is difficult to compare these reported incidences.

2.2. Indirect contamination of milk from the farm environment

L. monocytogenes is widespread in the farm environment. Fox et al. (2009) found that an average of 19% (57/298) of farm samples were contaminated, while Latorre et al. (2009)

found *L. monocytogenes* in 7.3% (22/303) of samples. Many foods originate at the farm so there is potential for cross-contamination of food with *L. monocytogenes*.
Milk is one of the foods that originate on the farm and while pasteurisation will inactivate *L. monocytogenes*, consumption of raw milk, especially by farm families is widespread. In addition, there are many producers of artisanal dairy products, including ice cream, cheese, yoghurt, using unpasteurised milk and increasing interest at farm level in selling unpasteurised milk for consumption (Oliver et al. 2009).

2.3. Direct contamination of milk from animals

Infection by *L. monocytogenes* has been confirmed in a wide variety of host animals including more than 40 species of domestic and wild animals. The most susceptible domestic species are sheep, goats and cattle. Listeriosis manifests itself clinically in ruminants as neo-natal mortality (abortion), septicaemia and most commonly as encephalitis. In general, small numbers are affected (8-10% of the herd) with the animals surviving from 4 to14 days. In animals, susceptibility to infection with *L. monocytogenes* has been attributed to decreased cell-mediated immunity associated with advanced pregnancy (Quinn et al. 2011).
Udder infection with *L. monocytogenes* is most commonly reported in sheep and goats (Low and Donachie 1997). *L. monocytogenes* bovine mastitis is less commonly reported where sub-clinical mastitis in cows can go undetected (Gitter et al. 1980; Van Daelen and Jaartsveld 1988; Fedio et al. 1990; Bourry et al. 1995; Schoder et al. 2003; Winter et al. 2004). In such cases, milk often remains visually unchanged, and with no clinical signs of infection, contamination can persist even after treatment (Fedio et al. 1990).
A recent case study demonstrated direct excretion of *L. monocytogenes* into cow's milk (Hunt et al. 2012). Levels of hygiene on the farm involved in that study were visually very good, and all non-milk contact dairy environmental swabs were negative, indicating the source of contamination was direct excretion into the milk. Following a visual inspection, none of the cows in the herd had any physical signs of infection, but an indistinguishable *L. monocytogenes* isolated was obtained from one cow and from the milk.

2.4. Growth in milk and raw milk consumption

L. monocytogenes has the ability to grow at 4°C and therefore has the potential to grow in bulk-tank milk, or during the distribution of unpasteurised milk. A risk assessment study has shown that a greater risk of listeriosis was associated with consumption of unpasteurised milk obtained from retail and farm stores as compared with unpasteurised milk obtained from bulk

tanks (Latorre et al. 2011). This was probably due to additional time-temperature combination steps in the retail and farm store models, which increased the chances for growth of *L. monocytogenes* in the unpasteurised milk. In Greece, a probabilistic model to evaluate the growth of *L. monocytogenes* during the chill chain of pasteurized milk (including transport, retail storage, and domestic storage) predicted that in 44.8% of the milk cartons released to the market, the pathogen could grow until the time of consumption (Koutsoumanis et al. 2010). However, based on an initial contamination level of 1 cell/1-liter carton, the predicted percentage of milk cartons in which the pathogen would exceed the safety criterion of 100 cells/ml at the time of consumption, was 0.14%. In addition, the milk environment and storage conditions have an influence on *L. monocytogenes* virulence. It has been shown that incubation in pasteurized milk at 4°C resulted in a higher invasion and intracellular proliferation of *L. monocytogenes* strains compared to raw milk when put into contact of human cells *in vitro* (Pricope-Ciolacu et al. 2013). The level of fat in milk also significantly affected the *in vitro* virulence of *L. monocytogenes* whereas the contents in caseins and lactose did not.

3. Occurrence in milk products

Lower prevalence of *L. monocytogenes* has been reported in dairy products than in other ready-to-eat foods. However, cheeses appear to be contaminated with *L. monocytogenes* more frequently than milk, and retail cheeses, particularly soft cheeses, have been found to be contaminated in several instances (Lianou and Sofos 2007).

3.1. Occurrence and growth in cheese

L. monocytogenes has been detected in a wide variety of cheeses all over the world and results for some of these surveys are presented in Table 2.

Even if the presence of *L. monocytogenes* is not detected in cheese, some authors consider that the presence of *Listeria* spp. other than *L. monocytogenes* indicates that hygiene whether at milking or during cheese making could be insufficient (Arrese and Arroyo-Izaga 2012).

In the EU, regulations specifically refer to the ability of food to support growth of *L. monocytogenes*, with maximum numbers of 100 cfu/g if food cannot support growth and absence if it can. Therefore, it is important to be able to assess the ability of food to support growth. Predictive modelling, based on experiments in laboratory media, can be used to make an initial assessment of growth ability, however the results from predictive modelling are not always accurate. Schvartzman et al. (2011) studied the effect of pH, water activity and

Table 2. Examples of occurrence of *L. monocytogenes* in cheese

Year	Country	Type of cheese	Type of milk	Heat treatment	No. positive samples/Total samples tested (%)	Origin of contamination	Reference
2012-2013	Ireland	Farmhouse Cheddar cheese	Cow	Raw	12/20 (60)	Yard (outside environment)	Dalmasso and Jordan (2013a)
2011	Jordan	Soft to semi-hard brined white cheese		Raw or pasteurisation	39/350 (11)		Osaili et al. (2012)
2011	Mexico	Fresh cheese	Cow	Raw	18/200 (9)		Torres-Vitela et al. (2012)
2010	Croatia	Fresh cheese	Cow		2/60 (3)		Frece et al. (2010)
2007	Italy	Gorgonzola, blue-veined mould-ripened cheese	Cow	Pasteurisation	2/18 (11)	Maturing shelves	Cocolin et al. (2009)
2007	Ireland	Farmhouse cheeses mould ripened, blue mould, smear-ripened, fresh, semi-soft and hard cheeses)	Cow, goat, ewe	Raw or pasteurisation	21/351 (6)		O'Brien et al. (2009)
2005	Brazil	Minas frescal cheese, Latin-style soft cheese	Cow	Pasteurisation	6/10 (60)	Storage coolers	Brito et al. (2008)
2005	UK	Unripened, ripened soft and semi-hard cheeses	Cow, goat, ewe, other	Pasteurisation	4/2618 (0.15)		Little et al. (2008)
2004	UK	Unripened, ripened soft and semi-hard cheeses	Cow, goat, ewe, other	Raw or thermization	17/1819 (1)		Little et al. (2008)
1991	England and Wales	Soft, ripened cheese	Cow	Pasteurisation	63/769 (8.2)		Greenwood et al. (1991)
1989	Italy	Soft cheese	Cow	Raw milk	2/21 (1.6)		Massa et al. (1990)

inoculum level on the growth/no growth boundary of *L. monocytogenes* in cheese. They showed that in 40% of cases, predictive modelling did not accurately predict growth. Similar results were obtained by Rosshaug et al. (2012). Therefore, challenge studies to determine the ability of a food to support growth of *L. monocytogenes* are essential. Thus, D'Amico et al. (2008) demonstrated that a 60-day period of ripening did not prevent the growth of *L. monocytogenes* in artificially contaminated surface-mold-ripened soft cheeses made from raw milk. Alternatively, growth in naturally contaminated cheese can be studied. Dalmasso and Jordan (2013a) determined that there was no growth in Cheddar cheese naturally contaminated with < 10 cfu/g of *L. monocytogenes*.

3.2. Occurrence in yoghurt and other fermented milk products

Fresh or traditional milk products obtained from unpasteurised milk in many countries are highly susceptible to *L. monocytogenes* and have the potential to support its growth until consumption of the products. *L. monocytogenes* has been shown to survive in products such as cultured buttermilk, butter, and even yoghurt (Choi et al. 1988; Farber and Peterkin 1991) (Table 3). (Kassaify et al. 2010) reported the occurrence of *L. monocytogenes* in 42% of a Middle Eastern coagulated cream products.

Even if fermentation processes can prevent *L. monocytogenes* from growing in fermented milk products, it has also been shown that some strains can adapt to acid conditions and that acid adaptation enhances the survival of *L. monocytogenes* in acidified dairy products, including cottage cheese, yoghurt, and whole-fat Cheddar cheese (Gahan et al. 1996). One mechanism employed by *L. monocytogenes* for survival at low pH is the adaptive acid tolerance response where a short adaptive period at a nonlethal pH induces metabolic changes that allow the organism to survive a lethal pH (Smith et al. 2012). Such survival of acid conditions by *L. monocytogenes* is likely to involve a variety of regulatory responses.

4. *L. monocytogenes* in the processing environment

L. monocytogenes is ubiquitous in the environment (Fox et al. 2011a) and has been isolated from a wide variety of ready-to-eat foods, not only from dairy products but also from meat and fish products, among others (Lianou and Sofos 2007). Its occurrence in the environment may pose the threat of *L. monocytogenes* transfer from the environment to the milk product, even though the routes of contamination are not always clearly identified. Although the control of *L. monocytogenes* in food is essential, it is also crucial to control *L. monocytogenes* occurrence in food processing environment to avoid cross-contamination (Fig. 2).

Table 3. Examples of occurrence of *L. monocytogenes* in milk and milk products (cheese not included)

Year	Country	Type of milk product	Heat treatment	Type of milk	No. positive samples/Total samples tested (%)	Reference
2010	Croatia	Cream of raw milk	Raw	Cow	5/60 (8.3)	Frece et al. (2010)
2010	Croatia	Milk	Raw	Cow	4/60 (6)	Frece et al. (2010)
2009-2010	USA	Milk	Raw	Cow	107/21(4 50)	Jackson et al. (2012)
2007-2008	New Zealand	Milk	Raw	Cow	2/295 (0.7)	Hill et al. (2012)
2007	Lebanon	Qishta, Middle Eastern coagulated cream product	Powdered or pasteurized liquid milk	Cow	13/31 (42)	Kassaify et al. (2010)
2003-2004	Algeria	Milk	Raw	Cow	10/233 (4)	Hamdi et al. (2007)
2002-2003	Portugal	Milk	Raw		2 /105 (2)	Kongo et al. (2006)
2001-2002	USA	Milk	Raw	Cow	2/248 (0.8)	Jayarao et al. (2006)
1991	England and Wales	Milk	Pasteurization	Cow	(1.1)	Greenwood et al. (1991)

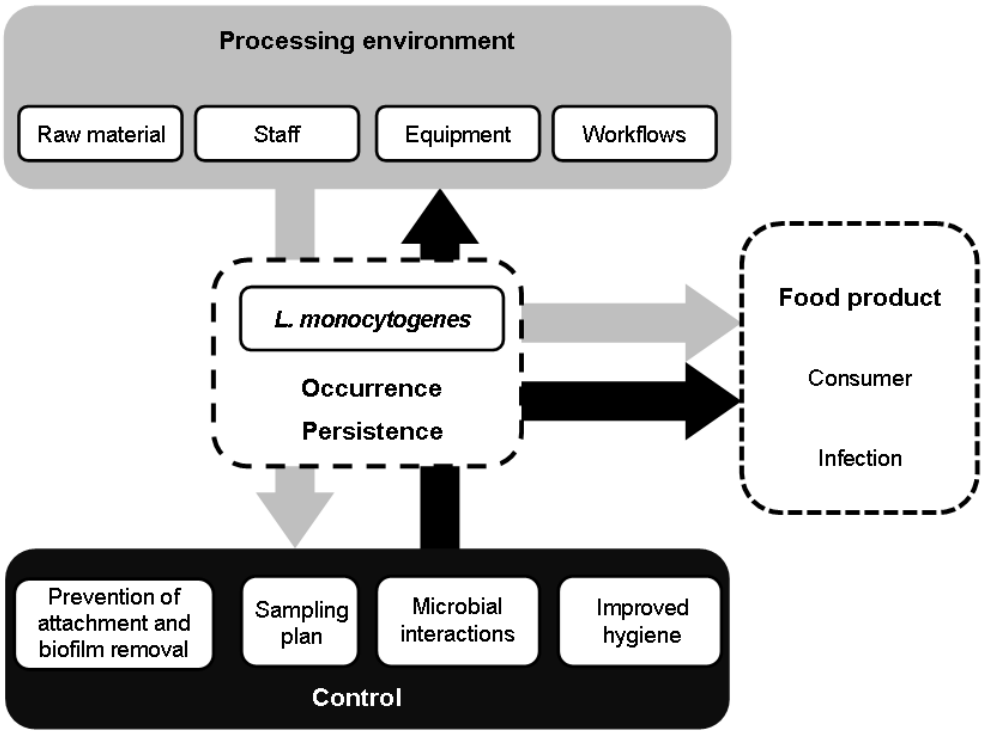

Fig. 2. Flow-chart of contamination of milk product processing environment with *L. monocytogenes*.

4.1 Occurrence in the processing environment

In many cases the foods are contaminated at the processing facilities e.g. at the slicing or packaging step, as *L. monocytogenes* has a remarkable ability to survive and persist in food processing environments. The bacterium is ubiquitous in the environment and may therefore be transmitted into the processing facilities by the raw materials, the workers, trucks, tools, cleaning materials or machines (Reij et al. 2004) or it may hide in the factory and survive the cleaning and disinfection procedures, forming biofilms and then occasionally cross contaminating food products.

In cheese processing facilities with associated dairy farms it is essential that physical barriers, such as an efficient footbath, exist between the external environment and the inside of the processing facility, as the dairy farm environment is known to be a potential source of *L. monocytogenes* contamination (Ho et al. 2007; Fox et al. 2009). Non-food contact surfaces, such as drains, cracks on floors, wheels of mobile equipment, etc, in cheesemaking facilities and outside the processing area have been reported as the main risk to food contamination

with *L. monocytogenes* (Dalmasso and Jordan 2013a), which indicate that efforts to reduce processing environment contamination are worthwhile.

Risk assessment analysis for *L. monocytogenes* in the dairy sector is increasingly being used to help understand and control the risks and ensure food safety. For example, a qualitative risk assessment for microbial hazards in Swiss dairy products that considered multiple hazards and products from different types of dairies along the food chain has recently been described (Menéndez González et al. 2011). The results obtained helped to make recommendations on the design of the national risk-based monitoring programme of dairy products. In the case of the production of a soft cheese made from pasteurised milk, a quantitative risk assessment analysis helped to identify the main factors and effects having an impact on the final risk, to determine intervention strategies at different food processing steps and to orientate specific research (Tenenhaus-Aziza et al. 2013).

4.2 Tools to monitor routes of contamination

Subtyping of *L. monocytogenes* strains is essential to identify isolate relatedness and track routes of contamination throughout the food system, which is very important for developing control strategies. It also provides insights into the ecology of *L. monocytogenes*, the population genetics and epidemiology (Wiedmann 2002). Several subtyping methods, not exclusively dedicated to monitoring routes of contamination like serotyping, ribotyping, phage typing or MLST, exist and have been recently reviewed (Jadhav et al. 2012). This section specifically focuses on some methods used to track *L. monocytogenes* strains and to understand routes of contamination throughout the processing environment.

4.2.1 PFGE

Pulsed-Field Gel Electrophoresis (PFGE) is the ‘gold standard’ method for assessing *L. monocytogenes* strain interrelatedness (Goering 2010) and for monitoring the putative routes of contamination in food processing environments (Fox et al. 2011a). PFGE was first developed by Schwartz and Cantor at Columbia University in 1984 (Schwartz and Cantor 1984). It has made possible the separation of large DNA fragments. In conventional agarose gel electrophoresis, DNA molecules bigger than 40-50 kb in size fail to migrate efficiently and appear in a gel as a single large diffuse band due to their size-independent co-migration known as reputation (Singh et al. 2006; Slater 2009). PFGE is based on periodical changes in the direction of the electrical field, termed pulsed-field, during migration of DNA in an agarose gel and allows the separation of DNA molecules over 1,000 kbp. Briefly, the

principal of PFGE is as follows: bacteria are embedded in agarose plugs in which cells are lysed in order to release DNA into the agarose plug. DNA is then digested and cleaved into large fragments by restriction enzymes and separated in a horizontal agarose gel using pulsed-field migration. This results in DNA fragment patterns or pulsotypes which differ from one strain to the other depending on the number and size of DNA fragments obtained from the digestion. Pulsotypes are used to identify and to compare bacterial strains such as *L. monocytogenes* after analysis of the gel image using specific software. The PulseNet International network proposes several standardized PFGE protocols for the study of foodborne pathogenic bacteria including *L. monocytogenes* (PulseNet USA 2009). This allows the creation of databases for the comparison of strains worldwide (Martin et al. 2006; Fox et al. 2012).

For example, PFGE was used to identify the route of contamination of farmhouse Cheddar cheese from the outside to the inside of the cheese processing facility and finally to the cheese (Dalmasso and Jordan 2013a). The transfer of *L. monocytogenes* strains from dairy farms to associated cheese processing facility has also been demonstrated using PFGE (Ho et al. 2007).

4.2.2 Random amplified polymorphic DNA (RAPD)

The RAPD method is based on the amplification of unspecified genomic sequences with arbitrary sequences used as single primers in order to generate a band profile. The main weakness of RAPD is that it has a low reproducibility because of the low annealing temperatures used in the PCR protocol which may generate different amplification patterns in different laboratories (Tyler et al. 1997; Jadhav et al. 2012). Although less discriminative than PFGE, RAPD is a cost-effective, rapid and useful method for comparing strains (Gravesen et al. 2000). It has been widely used to type dairy isolates. For example, Chambel et al. (2007) used RAPD to compare more than 200 strains from Portuguese dairy factories and evaluate the pattern of contamination throughout time.

4.2.3 Other methods for tracking strains

Amplified fragment length polymorphism (AFLP), and fluorescent AFLP (fAFLP), are based on the digestion of the genomic DNA by restriction enzymes, followed by PCR amplification and capillary gel electrophoresis (Graves et al. 2007; Roussel et al. 2013). Lomonaco et al. (2011) compared two AFLP methods and PFGE in regard to discriminatory power, typeability and concordance. They showed a very similar discriminatory power between the

two AFLP methods and PFGE and suggested that AFLP may be used as part of routine surveillance in production plants in order to reveal sources and routes of transmission.
REP (Repetitive Element Palindromic) and ERIC (Repetitive Intergenic Consensus) repeats are imperfect palindrome sequences in bacterial genomes which can form stem and loop structures. REP palindromic sequences are found in the extragenic regions of the genome in direct or reverse orientation whereas ERIC sequences generally consist of a central highly conserved inverted repeat sequences (Jadhav et al. 2012). REP- and ERIC-PCR are based on the amplification by PCR of these small sequences resulting in REP- or ERIC-PCR band patterns that are used to compare strains depending on the size and the number of bands on the profile. REP-PCR subtyping of *L. monocytogenes* has a lower discrimination power than PFGE but is still a fast low-cost molecular subtyping method for the routine monitoring of *L. monocytogenes* in processing environments and could present an advantageous complement to PFGE (Zunabovic et al. 2012).

4.3 Persistence in the processing environment

Persistence means that particular types of microorganisms survive for prolonged periods of time in certain habitats. The mechanisms of persistence are the topic of much interest and debate and have been largely reviewed (Carpentier and Cerf 2011; Halberg Larsen et al. 2013). Persistence of *L. monocytogenes* relies on many factors, such as the physical and microbial natural habitat, transmission routes and genetic determinants. Owing to its elaborate physiological adaptation mechanisms, *L. monocytogenes* can survive and even proliferate under adverse environmental conditions such as low pH, high salinity and low temperature (Khelef et al. 2006).
In addition to strains persisting at larger scale cheese production facilities (Lomonaco et al. 2009), persistence has also been documented at smaller artisan facilities (Fox et al. 2011a; Dalmasso and Jordan 2013a, b).
Various factors have been studied for their role in the persistence of *L. monocytogenes* strains, including disinfectant and desiccation resistance (Aase et al. 2000; Holah et al. 2002; Kastbjerg and Gram 2009; Vogel et al. 2010), differences in gene expression (Fox et al. 2011b) and biofilm formation (Norwood and Gilmour 1999; Lunden et al. 2000; Djordjevic et al. 2002).

4.3.1. Disinfectant tolerance

It has been suggested that persistence could be due to harbourage sites that are not sufficiently sanitised, and thus lead to recontamination events by strains resident in these sites (Carpentier and Cerf 2011). These sites include slots, drains, slicers, conveyer belts, and packaging machines. Inadequate cleaning and disinfection procedures or other factors like food debris and biofilm formation can significantly reduce the efficiency of disinfectants against *L. monocytogenes* and consequently contribute to the maintenance of some strains in the processing environment. Some persistent strains have been shown to be more resistant to disinfectants such as quaternary ammonium compounds (QACs) than non-persistent strains (Aase et al. 2000; Lunden et al. 2003; Fox et al. 2011b). This could be due to the presence of genetic elements in persistent strains such as a plasmid-based gene cassette that confers increased resistance to a commonly used QAC compounds such as benzalkonium chloride (Elhanafi et al. 2010). It has also been shown that sublethal concentrations of benzalkonium chloride increased the proliferation of *L. monocytogenes* in the host cell (Pricope et al. 2013).

4.3.2 Attachment and biofilm formation

The attachment of *L. monocytogenes* cells to surfaces and the formation of biofilms in the food processing environment is a well known phenomenon and has been recently reviewed (Valderrama and Cutter 2013). Biofilm formation is presumed to protect bacteria against environmental stresses (Ronner and Wong 1993) and so to facilitate persistence. For example, the examination of strains from bulk milk and milking equipment, and examination of biofilm on the milking equipment in a dairy farm, supported the view that the ability of *L. monocytogenes* to form biofilm is important in persistence of strains (Latorre et al. 2009). Persistent strains are believed to have better attachment abilities than non-persistent strains (Norwood and Gilmour 1999) even if in some cases, some non-persistent strains have shown a high aptitude to colonize surfaces (Lunden et al. 2000). A recent study also found that persistent strains from dairy environment demonstrated better adherence than sporadic strains (Latorre et al. 2011). Higher biofilm formation among persistent compared to non-persistent strains from bulk milk samples was also described (Borucki et al. 2003). Food composition also seems to influence the adhesion of *L. monocytogenes* to solid surfaces during dynamic flow conditions (Skovager et al. 2013).

Moreover, multispecies biofilms, as commonly found, are believed to increase the protection of pathogenic bacteria, and especially *L. monocytogenes*, against disinfection (Norwood and Gilmour 2000; Van der Veen and Abee 2011). The presence of bacteria producing

extracellular polysaccharide inside the biofilm may play a role in *L. monocytogenes* persistence by limiting the efficacy of sanitizers (Bremer et al. 2001; Carpentier and Cerf 2011).

4.3.3 Other factors potentially involved in persistence

Another phenomenon believed to contribute to persistence is the possible internalisation of *L. monocytogenes* inside protozoa, which can encyst to survive harsh conditions and thus protect *L. monocytogenes* from hostile environment (Greub and Raoult 2004). Indeed, *L. monocytogenes* has been shown to survive after ingestion by protozoa and to be released after lysis of the protozoa sometime later (Ly and Muller 1990). However, there is some limitation of this mechanism as it has been demonstrated that *L. monocytogenes* was unable to persist in *Acantamoeba* (Doyscher et al. 2013).

Integration of prophage DNA into the *com*K gene of *L. monocytogenes* was also proposed to lead to the persistent phenotype (Verghese et al. 2011). Persistent strains also seem to up-regulate genes implicated in the utilisation of carbon sources, possibly conferring to these strains competitive advantage, and thus promoting persistence (Fox et al. 2011b).

Persistence of *L. monocytogenes* is a complex issue and could possibly be due to the contribution of different factors, which can vary from strain to strain (Fox et al. 2011b).

5 Control of *L. monocytogenes* in the processing environment

The control of *L. monocytogenes* in the processing environment is essential in contributing to food safety. The challenge for food manufacturers is to direct efforts to prevent the entry and establishment of *L. monocytogenes* within the processing environment. Good Manufacturing Practices (GMP) and employee training will facilitate this. In addition, an adequate Hazard Analysis Critical Control Plan (HACCP), or similar type plan, is necessary

5.1 Sampling plan

Environmental sampling is an effective way to assess hygiene and prevent future contamination events (Tompkin 2002). It is important to focus sampling to sites where occurrence of *L. monocytogenes* is expected or where it may be present and may contaminate food. Published guidelines exist for processing environment sampling (European Union Reference Laboratory for *Listeria monocytogenes* 2012). In their work, Dalmasso and Jordan (2013b) have demonstrated that processing environment sampling plans in a cheese processing facility were effective to assess hygiene, and implement corrective actions in order to prevent contamination events, limit *L. monocytogenes* occurrence in food processing

facilities, and consequently assure the safety of the cheese. Being aware of the occurrence of *L. monocytogenes* in the processing environment is important as this occurrence can be dealt with. Having an action plan in the case of a positive result from sampling as part of the hygiene procedures is essential (Halberg Larsen et al. 2013).

5.2 Prevention of attachment and biofilm removal

In the prevention of bacterial attachment to surfaces and biofilm formation, the choice of material regarding the physical properties of their surface is crucial as persistence is increased with porous materials, which should therefore be avoided (Mead and Scott 1994). Stainless steel has proven to be a material with low adherence of bacteria and easy to clean (Midelet and Carpentier 2002; Somers and Wong 2004). Many materials have incorporated bacteriostatic or bacteriocidal agents which can reduce the attachment of bacteria to surfaces. However, these properties can be diminished or impaired with the presence of protein residues (Chaitiemwong et al. 2010; Møretrø et al. 2011).

Before any step of disinfection, a cleaning procedure has to be carried out. It contributes to removal of biofilm. A robust disinfection routine should include alternation between chemical disinfectants with different properties and mechanisms of action, or between chemical and physical disinfection such as heat or UV. Standardized tests have been defined in order to ensure that disinfectants meet the required efficacy criteria (CEN 1997; 2002). As the composition of biofilm, the cleaning and disinfection procedures used and the compounds used vary from one food company another, there is currently no ideal/standard method for the complete removal of biofilms, as shown in several studies (Belessi et al. 2011; Cruz and Fletcher 2012).

5.3 Microbial interactions

Microbial interactions have great potential to improve safety in the food chain and thus reduce the associated public health risk. These microbial interactions have been extensively reviewed (Jordan et al. 2013). Fermentation processes involved in the production of cheese and yoghurt are known to have antimicrobial properties against food spoilage microorganisms but also pathogens such as *L. monocytogenes*. Therefore, using traditional food fermentation processes with potentially hazardous raw materials (like raw milk) may be used for production of food with improved quality and increased safety (Adams and Mitchell 2002). Many studies have shown that lactic acid bacteria (LAB) involved in fermentation processes can control and inhibit pathogenic bacteria by different mechanisms like nutrient competition,

immunostimulation, competition for binding sites and production of antimicrobial substances such as organic acids or hydrogen peroxide. Moreover, some LAB can also produce proteinaceous bacterial toxins, bacteriocins, which can be used as natural antibacterial preservatives for food (Gálvez et al. 2007). LAB bacteriocins usually exhibit activity against Gram-positive pathogens such as *L. monocytogenes* (Sobrino-Lopez and Martin-Belloso 2008) and have proven to be effective in food (Mills et al. 2011). Currently, two LAB bacteriocins, nisin and pediocin PA-1, are commercially available and have applications in food systems (EFSA 2006). Research on the use of bacteriocins in the food chain has been extensively carried out (Cotter et al. 2005) and developments in bacteriocins continue.
The use of bacteriophages for control of *L. monocytogenes,* although not strictly microbial interaction, is also of importance. Bacteriophages are good candidates for use as natural preservatives because of their ability to specifically target their host bacterium, their self-perpetuation and their stability during prolonged storage (Coffey et al. 2010). In dairy products, bacteriophages have been successfully used to control the presence and reduce the growth of *L. monocytogenes* (Greer 2005). In the US, the Food and Drug Administration approved the use of a bacteriophage mixture that could be sprayed on specific food products to reduce the presence of *L. monocytogenes*, under the category of "food additives". A similar product can be used in the EU, but under the category 'processing aid'.

6. Concluding remarks and future perspectives

More knowledge on the *L. monocytogenes* strains contaminating processing facilities and the genetic and physiological factors that allow persistence is needed. To obtain this, more relevant models simulating niches in the food production environments combined with analytical tools to investigate the composition as well as genetic and physiological responses of complex microbiota should be developed (Malley et al. 2013). This will provide more relevant background information needed to develop new eradication strategies. Examples of new principles for combating biofilms that could be further investigated include the use of bacteriophages, targeting cell-to-cell communication, or the iron pathway or enzymes attaching specific biofilm components or new antibacterial materials. With new and fast techniques for strain characterization, differentiation of *L. monocytogenes* strains with respect to virulence will be possible. Thus, virulence of a particular isolate may be taken into account in risk assessment.

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